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Secretion of the Notch-1 Aβ-like Peptide during Notch Signaling*[S]

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The canonical pathway of Notch signaling is mediated by regulated intramembrane proteolysis (RIP). In the pathway, ligand binding results in sequential proteolysis of the Notch receptor, and presenilin (PS)-dependent intramembrane proteolysis at the interface between the membrane and cytosol liberates the Notch-1 intracellular domain (NICD), a transcription modifier. Because the degradation of the Notch-1 transmembrane domain is thought to require an additional cleavage near the middle of the transmembrane domain, extracellular small peptides (Notch-1 Aβ-like peptide (Nβ)) should be produced. Here we showed that Nβ species are indeed secreted during the process of Notch signaling. We identified mainly two distinct molecular species of novel Nβ, Nβ21 and C-terminally elongated Nβ25, which were produced in an ~5:1 ratio. This process is reminiscent of the production of Alzheimer disease-associated Aβ. PS pathogenic mutants increased the production of the longer species of Aβ (Aβ42) from β-amyloid protein precursor. We revealed that several Alzheimer disease mutants also cause a parallel increase in the secretion of the longer form of Nβ. Strikingly, chemicals that modify the Aβ42 level caused parallel changes in the Nβ25 level. These results demonstrated that the characteristics of C-terminal elongation of Nβ and Aβ are almost identical. In addition, because many other type 1 membrane-bound receptors release intracellular domains by PS-dependent intramembrane proteolysis, we suspect that the release of Aβ- or Nβ-like peptides is a common feature of the proteolysis during RIP signaling. We anticipate that this study will open the door to searches for markers of RIP signaling and surrogate markers for Aβ42 production.

Notch signaling is involved in cell differentiation as well as neurodegeneration (1). The canonical pathway for Notch signaling is mediated by regulated intramembrane proteolysis (RIP)[2] (2). In classical signaling, ligand binding to receptors induces downstream intracellular signals, such as Ca^{2+} influx and protein tyrosine phosphorylation. However, RIP signaling is distinct; the receptor is cleaved, liberating an intracellular fragment from the membrane that translocates to the nucleus where it modifies the transcription of target genes (3, 4).

In general, because both the ligand and the receptor for Notch signaling are transmembrane proteins, and signaling occurs only between neighboring cells, a process also called "local cell signaling" (5–7). Thus, although connections between Notch signaling and tumorigenesis have been reported (8, 9), studies to identify secreted molecules that reflect the level of Notch signaling have not been carried out. As a result, the only widely used method for measuring Notch signaling in living cells has been to employ intracellular reporter plasmids (10, 11).

Upon synthesis, the Notch-1 receptor undergoes furin-like cleavage at site (S) 1, forming a heterodimer that is expressed on the plasma membrane with the cleaved fragments (12, 13). The binding of ligands, such as DSL family proteins, at the plasma membrane induces endoproteolysis of Notch-1 by the ADAM/TACE/Kuzbanian family (14–16). This cleavage occurs at S2 within the extracellular juxtamembrane region and results in shedding of the heterodimerized Notch receptor (14–16). The resulting transmembrane fragment, referred to as Notch extracellular truncation, undergoes constitutive presenilin (PS)-dependent intramembrane proteolysis at S3 and S4, which we refer to as "dual-intramembrane proteolysis" (17–19). The S3 cleavage occurs at the interface between the membrane and cytosol, thus determining the N terminus of NICD (17, 18). The presence of the S4 cleavage near the middle of the transmembrane domain (19, 20) and FLAG-tagged Nβ (19) suggests that a putative Notch-1 fragment (Nβ) is produced during the sequential proteolysis of Notch receptors. However, amino acid sequences of these Notch peptides and the composition of their molecular species remain unknown. Moreover, whether they are secreted during Notch signaling has not been determined.

Like Notch receptors, β-amyloid protein precursor (βAPP) undergoes PS-dependent "dual-intramembrane proteolysis" at γ and ε sites (21). γ-Cleavage, which corresponds to S4 cleavage in Notch-1, results in the secretion of Alzheimer disease (AD)-associated Aβ (22), whereas ε-cleavage, corresponding to the S3 cleavage site in Notch-1, releases the βAPP intracellular domain, which has been suggested to regulate transcription

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[2] The abbreviations used are: RIP, regulated intramembrane proteolysis; Aβ, amyloid-β peptide; AD, Alzheimer disease; βAPP, β-amyloid protein precursor; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; N1CS, Notch-1 Lin/Notch Repeat CC > SS; NSAIDs, nonsteroidal anti-inflammatory drugs; NICD, Notch-1 intracellular cytoplasmic domains; MS, mass spectroscopy; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; Nβ, Notch-1 Aβ-like peptide; PS, presenilin; compound W, 3,5-bis(4-nitrophenoxy)benzoic acid; sw, Swedish; WT, wild type; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; CW, Compound W; PMA, phorbol 12-myristate 13-acetate; CHO, Chinese hamster ovary; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine *t*-butyl ester.

(21, 23, 24). In PS-dependent proteolysis, there is some diversity in the specific sites of cleavage (19, 22, 25).[3] This is an important aspect of PS-dependent proteolysis because the pathological molecule Aβ42 is generated by cleavage of βAPP at γ42, a variant of the γ-cleavage site (22). Generally, Aβ42 accounts for ~10% of Aβ production, and most familial AD-associated PS1/2 or βAPP mutants show an increase in Aβ42 production, a peptide that plays an causative role in AD pathology (22). Although Aβ42 induces the formation of senile plaque both in familial and sporadic AD brains, the high degree of Aβ42 aggregation makes it difficult to determine whether Aβ42 generation is enhanced in the pathogenesis of sporadic AD. Therefore, a surrogate marker that precisely reflects the level of Aβ42 generation could be useful.

Elucidation of the mechanism of Aβ42 generation, *i.e.* how the γ-cleavage diversity occurs, is essential because a small change in the precision of γ-cleavage is sufficient to lead to AD pathology. PS-mediated intramembrane proteolysis is known to occur in many other type 1 transmembrane receptors at a site near the cytosolic interface of the transmembrane domains, corresponding to S3/ε cleavage (26, 27). Also, cleavage corresponding to the S4/γ site, which lies in the middle of the transmembrane domain, has been proposed for Notch-1, βAPP, and CD44 (19, 28). Therefore, whether γ-like cleavage occurs in all the substrates or whether there is any diversity among cleavage sites remains unknown. Also, certain chemicals, such as some nonsteroidal anti-inflammatory drugs (NSAIDs), affect the precision of γ-cleavage, increasing or decreasing the generation of Aβ42 (29, 30), but whether this effect is specific to βAPP or is shared by other substrates has not been determined.

To investigate the mechanism of Nβ secretion during Notch signaling, we utilized mouse Notch-1 and its derivatives as substrates. We found that upon ligand binding full-length Notch-1 undergoes endoproteolysis, which transmits Notch signaling and simultaneously secrets untagged Nβ species. These Nβ species shared a common N terminus (derived from S2 cleavage) but had distinct C termini (derived from S4 cleavage). Interestingly, in many cases, when the relative level of pathological Aβ42 was altered by expression of PS mutants or addition of certain chemicals, the relative level of Nβ25, a major species of longer Nβ, changed concomitantly. These results suggested that Nβ is secreted during Notch signaling and that the proteolytic processes determining the C termini of Nβ and Aβ are very similar.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The polyclonal antibody 6521 and the rat monoclonal antibody 5E9 were raised against a synthetic peptide (VKSEPVEPPLPSQ) corresponding to the N terminus of Notch extracellular truncation using methods described previously (31). Antibodies 9E10 against the c-Myc epitope, 4G8 against the Aβ peptide, and H114 against the C terminus of Jagged-1 were purchased. Synthetic peptides VKSEPVEPPLPSQLHLMYVAA (Nβ21) and VKSEPVEPPLPSQLHLMYVAAAAFV (Nβ25) were dissolved in Me_2SO or HFIP and stored at −80 °C (32). Upon use, the peptides in HFIP were dried using a Speed-Vac and dissolved in the appropriate solution. S2474 was synthesized by Shionogi and Co. Ltd. The γ-secretase inhibitors (L685,458 and DAPT), PMA, NSAIDs (sulindac sulfide, indomethacin, and naproxen), fenofibrate, farnesyl pyrophosphate ammonium salt, geranylgeranyl pyrophosphate ammonium salt, and Compound W (CW), 3,5-bis-(4-nitrophenoxy)benzoic acid were purchased.

cDNA Constructs—The pCS2 vector containing the cDNA for mouse Jagged-1 (kind gift from Dr. Rafael Kopan) (33) and the pTracerCMV containing in the mNotch-1 cDNA were described previously (kind gift from Dr. Jeffrey S. Nye) (34). The cDNAs encoding N1CS (mN1 LNR CC > SS) (kind gift from Dr. Rafael Kopan) (14) and Notch-1, in which the C-terminal 348 residues were replaced with a hexameric Myc tag (17), were cloned into the pcDNA3-hygro(+) vector. *HES-1*-luc (kind gift from Dr. Alain Israel) (10) and pGa981-6 constructs (kind gift from Dr. Georg W. Bornkamm) (11), which contain the firefly luciferase cDNA under control of the *HES-1* promoter and the hexamerized 50-bp EBNA2-response element of the *TP-1* promoter, respectively, were described previously. The pRL-TK construct was obtained from Promega.

Cell Culture and Cell Lines—HEK293 cells stably expressing βAPP sw and either WT or mutant PS1 were generated and cultured (19, 35). CHO cells stably expressing either WT PS1 or PS1 M146L were cultured as described previously (kind gift from Dr. Dennis J. Selkoe) (36). Cells stably expressing βAPP sw, PS derivatives, or both were maintained in media supplemented with 200 μg/ml G418, 200 μg/ml Zeocin, or both. Cells stably expressing Jagged-1, Notch-1, or N1CS were selected with 100 μg/ml hygromycin.

cDNA Transfection and Reporter Assay—To investigate the formation and function of Nβ, we stably transfected HeLa, CHO, or HEK293 cells with Jagged-1, N1CS, and Notch-1 cDNA constructs using Lipofectamine 2000 (Invitrogen). To study the release of Nβ during Notch signaling, HeLa or K293 cells in 8-cm dishes were transiently transfected with 5 μg of plasmids encoding Notch ligands and/or receptors, 5 μg of plasmids encoding the firefly luciferase reporter (*HES-1*-luc or pGa981–6), and 50 ng of the control *Renilla* luciferase reporter plasmid pRL-TK. The next day, the media were replaced, and the cells were lysed with 1× Passive Lysis Buffer (500 μl) (Promega) and freeze-thaw treatment. The pulse-chase experiment to detect the Nβ level was carried out at the same time. Firefly and *Renilla* luciferase activities in the lysates were measured with a dual luciferase reporter assay system (Promega).

Pulse-Chase Experiment—The cells were treated for 2 h with 1 μM L685,458 or 1 μM DAPT to inhibit γ-secretase activity or 20 ng/ml PMA to increase the efficiency of S2 cleavage. Next, following starvation in methionine-free minimum Eagle's medium, the cells were metabolically labeled overnight with 450 μCi of [^{35}S]methionine/cysteine (Redivue Promix; Amersham Biosciences) containing 5% dialyzed (*i.e.* amino acid-free) fetal calf serum and γ-secretase inhibitors or PMA. NSAIDs, compound W, fenofibrate, farnesyl pyrophosphate ammonium salt, and geranylgeranyl pyrophosphate ammonium salt were added to the media throughout the pulse periods.

Immunoprecipitation/Immunoblotting or Immunoprecipitation/Autoradiography—Collected conditioned media were adjusted to 50 mM Tris (from a stock solution of 1 M Tris, pH 7.4), 1:1000 protease inhibitor mixture (Sigma), and 5 mM EDTA (from a stock solution of 500 mM EDTA, pH 8.0). Next, the media were subjected to immunoprecipitation with antibodies 6521 or 4G8 for Nβ and Aβ, respectively (19). For Jagged-1 and Notch-1 derivatives, cell lysates were prepared (37) and analyzed by immunoprecipitation with antibodies H114 and 9E10, respectively (19). Proteins were separated by Tris-glycine (Invitrogen), Tris-Tricine (Invitrogen), or Tris-Bicine SDS-PAGE as described previously (38). The gels were fixed in 50% methanol, 10% acetic acid or 50% methanol, 5% glutaraldehyde (for Nβ). For immunoblotting, proteins were electrophoretically transferred to a polyvinylidene difluoride membrane and probed with the indicated antibodies (39). For autoradiography, the gels were dried and analyzed by fluorography (19).

[3] S. Tagami, A. Fukumori, and M. Okochi, manuscript in preparation.

Immunoprecipitation/MALDI-TOF MS—Immunoprecipitation followed by MALDI-TOF MS analysis was carried out as described previously (19). Briefly, cell lines were grown to confluence in 8-cm dishes. The culture media were replaced with media with or without 20 ng/ml PMA. After 6 h, the media were collected and immunoprecipitated by incubation for 8 h at 4 °C with antibody 6521. Following washing, immunoprecipitated peptides were eluted and analyzed by MALDI-TOF MS. Molecular masses and heights of the MS peaks were calibrated with angiotensin (Sigma), bovine insulin β-chain (Sigma), or both.

RESULTS

Notch-1 Peptides Are Secreted Extracellularly during Notch Signaling—Notch ligand binding to Notch receptors results in sequential proteolysis of the receptor, and PS-dependent intramembrane proteolysis at the interface between the membrane and cytosol (S3) liberates NICD that modifies the transcription of target genes (1) (Fig. 1*A*). Although it was shown that degradation of Notch-1 transmembrane domain requires additional proteolysis near the middle of the transmembrane domain (S4, dual cleavage) (19), secretion of extracellular small peptides (Notch-1 Aβ-like peptide; Nβ) during Notch signaling has not been determined (Fig. 1*A*). Therefore, we first examined whether sequential endoproteolysis of Notch receptors induced upon binding of Notch ligands (1) results in the secretion of Notch peptides. The full-length Notch-1 receptor does not undergo sequential endoproteolysis in the absence of ligand binding, because the S2 cleavage site is masked in the Notch-1 heterodimer formed by S1-cleaved Notch-1 fragments (1). In contrast, Notch-1 LNR CC > SS (N1CS) (Fig. 1*B*) inhibits heterodimerization of S1-cleaved Notch-1 (14). Thus, we expect that NICS shows constitutive consecutive S1, S2, S3, and S4 cleavage of Notch-1 that usually occurs depending on ligand binding (Fig. 1*B* and supplemental Fig. 1*A*).

To determine whether Notch-1 peptides are secreted, we metabolically labeled HEK293 cells stably expressing Notch-1 or N1CS with [^{35}S]methionine and then performed immunoprecipitation using antibody 6521, which binds to an epitope downstream of S2 (Fig. 1*B*). Most strikingly, autoradiography shows that N1CS-expressing cells secrete Notch-1 peptides of ~3 and 6 kDa, although they were present in different amounts (Fig. 1, *C*, *upper* and *lower panels*, and *D*). The Notch-1-expressing cells, however, produced only trace amounts of these peptides (Fig. 1*C*, *upper panel*). Moreover, addition of PMA to enhance the cleavage at S2 by matrix metalloproteases increased the level of released Notch-1 peptides by 4.73 ± 0.01-fold (Fig. 1, *C* and *D*). On the other hand, addition of γ-secretase inhibitors (L685,458 or DAPT) to inhibit dual-intramembrane proteolysis at S3 and S4 by PS-dependent endoproteolysis greatly reduced the amount of secreted Notch-1 peptide (Fig. 1*D* and supplemental Fig. 1*B*). These results indicated that the Notch-1 peptides are secreted in conjunction with the sequential proteolysis of the Notch-1 receptor and that the production of the Notch-1 peptides is associated with the efficiency of cleavage at S2 and S3/S4 by metalloproteases and PS-dependent proteases, respectively.

We next investigated whether the Notch-1 peptides are secreted during Notch signaling (Fig. 1*E*). Instead of N1CS, we used Notch-1 without any extracellular truncation and mutations (Fig. 1*B*) and observed Notch signaling mediated by Jagged-1 (a Notch ligand). We simultaneously measured the level of Notch signaling and analyzed whether Notch-1 peptides are secreted from cells expressing Jagged-1, Notch-1, or both (Fig. 1*E*). To determine the level of Notch signaling, we measured luciferase reporter activity in cells expressing *HES-1*-luc (10) (*3rd panel* in Fig. 1*E* and supplemental Fig. 2). We also examined the conditioned media for the presence of secreted Notch-1 peptides, as described in Fig. 1, *C* and *D*. We found that cotransfection with Jagged-1 and Notch-1 greatly increased the reporter activity (*3rd panel* of Fig. 1*E*), indicating substantial enhancement of transcription via the *HES-1* promoter. Most surprisingly, the secretion of Notch-1 peptides was also greatly enhanced in cells expressing both Jagged-1 and Notch-1, but it was hardly detectable in cells expressing either Jagged-1 (data not shown) or Notch-1 alone (Fig. 1*E*, *bottom panel*). These results indicated that the secretion of the Notch-1 peptides and the activation of Notch signaling are greatly increased when both Notch-1 and its ligand, Jagged-1, are present. In summary, sequential proteolysis of Notch-1 occurs upon Jagged-1 binding, leading to the production of not only NICD, which is released from the membrane to the cytosol (supplemental Fig. 1*B*), but also novel Notch-1 peptides that are released extracellularly (depicted in Fig. 1*A*).

Determination of the Amino Acid Sequences of the Notch-1 Peptides Reveals Secretion of Nβ—Next, we determined the amino acid sequences of the Notch-1 peptides secreted during the sequential endoproteolytic process of Notch-1 receptor (Fig. 2). The Notch-1 peptide species secreted by N1CS-expressing cells were immunoprecipitated with the 6521 antibodies, and their molecular masses were analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy (MS) (Fig. 2*A*). The spectrum of the Notch-1 peptides showed two major peaks with molecular masses of 2306 and 2694 Da. We determined the peptide sequences of these species based on that of the sequence surrounding the epitope for the 6521 antibody. Most strikingly, the Notch-1 peptides were found to be composed of peptides S2 to S4 (Table 1, supplemental Fig. 3, and supplemental Table 1). Finally, amino acid sequences of the Notch-1 peptides were determined by MALDI-TOF/TOF MS analysis (supplemental Fig. 3). Therefore, we found that these Notch-1 peptides correspond to the previously predicted Nβ (19). We named the shorter (2306 Da) and longer (2694 Da) Nβ species Nβ21 and Nβ25, respectively, where the number indicates the peptide length (Table 1).

We next synthesized Nβ21 and Nβ25 and analyzed them by MALDI-TOF MS. We confirmed that each peptide showed a single peak in the MS spectrum corresponding to the predicted molecular mass (Fig. 2*B*). We then examined the characteristics of each peptide on Tris-Tricine SDS-PAGE (Fig. 2*C*). We dissolved these peptides in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to prevent the formation of artificial oligomers (32) or in Me_2SO. In both cases, the migration of synthetic Nβ25 and Nβ21 approximately corresponded to 3- and 6-kDa bands, respectively (Fig. 2*C*, *left* and *middle panels*). These results showed that, unlike Nβ25 (or FLAG-tagged Nβ species (19)), Nβ21 may form an SDS-stable homodimer. Like Aβ, we detected extremely low levels of intracellular Nβ compared with secreted Nβ, but we did not detect higher molecular weight aggregates of secreted Nβ21 or Nβ25 on Tris-Tricine SDS-PAGE (data not shown). Also, the intensity of the ~6-kDa band of a mixture prepared with Nβ21 and Nβ25 species was not affected (Fig. 2*C*, *left panel*), indicating that the two Nβ species do not form a heterodimer. Thus, Nβ21 and Nβ25 are separated well by SDS-PAGE. Most interestingly, the migrations of secreted (Fig. 2*C*, *right panel*) and synthetic Nβ (Fig. 2*C*, *middle panel*) on SDS-PAGE were almost identical. Moreover, upon PS1 L166P mutant expression, the ~3-kDa Nβ band on SDS-PAGE and the MS peak corresponding to Nβ25 were predominantly detected (see Fig. 3, *A* and *C*). These findings indicate that Nβ21 and Nβ25 are the major types of secreted Nβ. Measurement of the radioactivity of the two bands indicated that ~20% as much Nβ25 is secreted from cultured cells as Nβ21 (Fig. 2*D*). Based on these results, it appears that the secreted Nβ species correspond to the

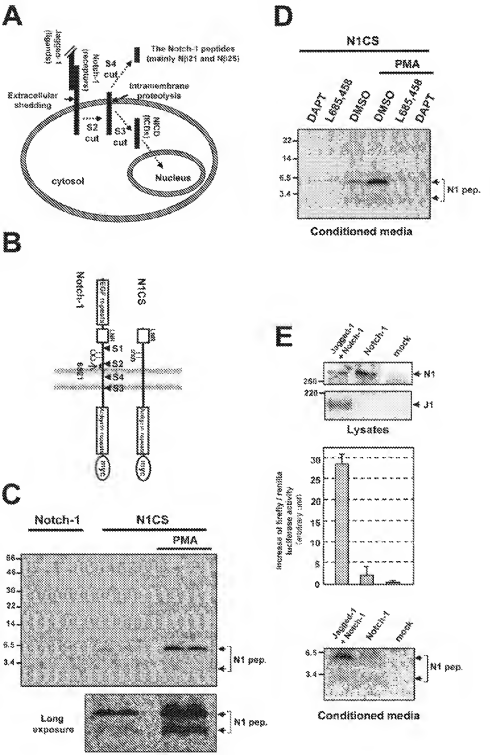

FIGURE 1. **Extracellular release of the Notch-1 peptides by sequential cleavage during Notch signaling.** *A*, schematic representation of the Notch-1 peptide (Nβ) release during canonical Notch signaling. Cleavage of Notch-1 at S4 results in the extracellular secretion of Nβ peptides (see below), whereas cleavage at S3 liberates NICD, which translocates to the nucleus to regulate target gene transcription. *B*, schematic representation of Notch-1 and N1 LNR CC > SS (N1CS), a peptide derived from Notch-1. S1 to S4 and the *gray* zone represent the sites of proteolysis and membrane, respectively. The Notch-1 and N1CS constructs both had C-terminal hexameric Myc tags. Note that the N1CS protein lacks the epidermal growth factor repeats present in Notch-1. Also, to avoid the formation of heterodimers following cleavage at S1, the cysteine residues between S1 and S2 were mutated to serine (14). The *arrow* indicates the recognition site for the 6521 antibody. *C*, immunoprecipitation/autoradiography analysis of Notch-1 peptides in conditioned media from cells treated without or with PMA. Notch-1 or N1CS-expressing cells were labeled by pulse-chase with [^{35}S]methionine. The Notch-1 peptides in conditioned media were separated by Tris-Tricine SDS-PAGE. The *lower panel*, which shows a longer exposure of the autoradiograph, confirms that without PMA treatment the Notch-1 peptides have two distinct molecular weights. Immunoprecipitation/immunoblotting analysis with a 9E10 antibody revealed that the levels of Notch-1 and N1CS expression were similar in the stably transfected cells (data not shown). *D*, immunoprecipitation/autoradiography analysis of conditioned media from N1CS-expressing cells treated with or without γ-secretase inhibitors. The γ-secretase inhibitors DAPT (1 μM) and L685,458 (1 μM) inhibited the secretion of the Notch-1 peptides. Similar results were obtained using HeLa cells expressing N1CS (data not shown). *DMSO*, Me$_2$SO. *E*, measurement of Notch signaling and the release of Notch-1 peptides in cells transfected with Jagged-1, Notch-1, or both. HEK293 cells were transiently transfected with the indicated constructs along with *HES-1*-luc and the pRL-TK dual luciferase reporter assay system. Empty plasmid vector was used as a mock transfection control. The results show cells expressing *HES-1*-luc. Similar results were obtained using cells expressing pGa981-6 (11) (data not shown). *Top panel*, to confirm the expression of Notch-1, immunoprecipitation/immunoblot analysis was carried out using anti-Myc antibody 9E10. *2nd panel*, Jagged-1 expression was determined using anti-Jagged-1 antibody H114. *3rd panel*, assay of Notch downstream signaling. A dual luciferase reporter assay was performed to compare the transcriptional activity of the *HES-1* promoter in each condition. *HES-1* promoter activity was measured as the relative ratio of firefly and *Renilla* luciferase activities. All values were corrected by the background luciferase activity in endogenous Notch-expressing cells transfected with *HES-1*-luc and pRL-TK. *Bottom panel*, conditioned medium from [^{35}S]methionine-labeled cells was analyzed by immunoprecipitation/autoradiography using antibody 6521.

sequence between S2 and S4 of Notch-1 and that Nβ is made up of two major species, Nβ21 and Nβ25, of which the former is predominant in cell culture (Fig. 2*D*).

Several Familial AD-associated PS1 Mutants Increase the Generation of Elongated Nβ as Well as the Pathological Elongated Aβ—Our results show that intramembrane cleavage of Notch-1 at S4 generates two major Nβ species. We therefore refer to these cleavages as S4-21 and S4-25 for Nβ21 and Nβ25, respectively (Fig. 3*D*). S4-25, the minor type of S4 cleavage, is located four amino acids C-terminal to S4-21, the major S4 cleavage. Like Notch-1, βAPP undergoes PS-dependent intramembrane proteolysis near the middle of the transmembrane domain (γ), resulting in the secretion of Aβ, which accumulates in AD brains (22). Topologically, the γ-cleavage site of βAPP corresponds to the S4 cleavage site of Notch-1 (19). Cleavage at γ42, an alternative form of γ-cleavage, generates Aβ42, which plays a causative role in the pathology of AD; therefore, understanding the mechanism of PS-de-

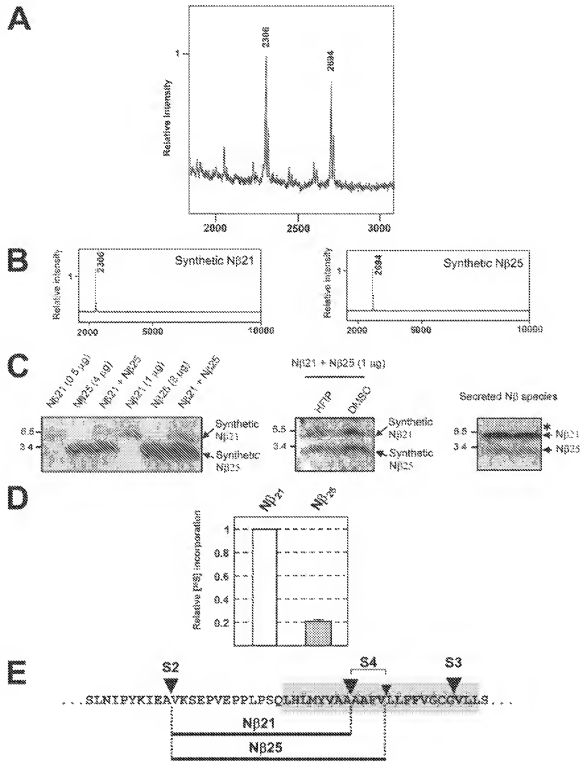

FIGURE 2. **Identification and characterization of secreted Nβ species.** *A*, determination of the molecular masses of the secreted Notch-1 peptides. N1CS-expressing cells were treated with 20 ng/ml PMA for 3 h, and the conditioned medium was analyzed by immunoprecipitation/MS analysis using antibody 6521. Shown is a representative MS spectrum. The MS spectrum was nearly identical for conditioned medium from cells transiently coexpressing Jagged-1 and Notch-1, although the peak heights were lower (data not shown). *B*, MALDI-TOF MS spectra of the synthetic Nβ21 and Nβ25. Although the synthetic Nβ21 appears as an ~6-kDa band on electrophoresis and may form an SDS-stable dimer (*C*), both the synthetic Nβ21 and Nβ25 were identified as single peaks in the MS analysis corresponding to monomeric forms. This shows that the mobility of Nβ21 on SDS-PAGE is lower than predicted for its molecular mass determined by MALDI-TOF MS. *C*, SDS-PAGE analysis of the synthetic Nβ21, synthetic Nβ25, and secreted Notch-1 peptides. *Left panel*, the synthetic Nβ21 and Nβ25 were separated by Tris-Tricine SDS-PAGE and analyzed by immunoblotting with antibody 5E9. *Center panel*, the synthetic Nβ21 and Nβ25 were dissolved in HFIP or Me_2SO and separated by Tris-Tricine SDS-PAGE. Because Aβ tends to aggregate, the synthetic Aβ was suspended in HFIP and then dried to obtain a monomeric Aβ solution (32). Like Aβ, Nβ contains a hydrophobic transmembrane domain sequence; therefore, we suspended the synthetic Nβ in HFIP to completely solubilize the peptides. We then examined whether the mobility on electrophoresis of the Nβ species was affected by the solvents. We were not able to detect the ~3-kDa monomeric Nβ21 band by electrophoresis. Note that similar intensities were observed for the Nβ21 or Nβ25 bands when equal amounts of the synthetic peptides were applied to the gel, indicating that the 5E9 antibody has similar affinities for the Nβ21 and Nβ25. *Right panel*, Nβ peptides in conditioned media from [^{35}S]methionine-labeled cells were separated by Tris-Tricine SDS-PAGE. The *asterisk* indicates a faint ~8-kDa band that was occasionally observed only in conditioned medium. The radioactivity of this band accounts for less than 5% of the total for all Nβ species, indicating a

TABLE 1
Major molecular species of the Notch-1 peptides in conditioned media

M_r (observed)	Peptide	Sequence	M_r (calculated)
2306	Nβ21	VKSEPVEPPLPSQLHLMYVAA	2306
2694	Nβ25	VKSEPVEPPLPSQLHLMYVAAAAFV	2694

pendent intramembrane endoproteolysis is an important issue in AD research (22).

To address the mechanism of cleavage at S4, which results in the production of Nβ, we next tested whether the precision of this cleavage varies simultaneously with the precision of γ-cleavage in βAPP. Most familial AD-associated PS mutants increase the relative level of secreted Aβ42 peptides, although the magnitude of the increase varies between mutants (22). We first tested whether the precision of S4 cleavage was affected by several types of PS mutants. Cells stably expressing the substrates sw mutant βAPP and N1CS, as well as WT or mutant forms PS1 (Fig. 3), were metabolically labeled with [^{35}S]methionine. Secreted Nβ or Aβ species were immunoprecipitated and separated by Tris-Tricine or Tris-Bicine (38) SDS-PAGE, respectively (Fig. 3*A*). To measure the efficiency of Nβ25 generation (S4-25 cleavage), we calculated the relative ratio of radioactivity of the Nβ25 band compared with the total for Nβ bands (including Nβ25; Fig. 3*B*). Using the same samples, we also determined the efficiency of Aβ42 generation (γ42 cleavage). We found that the conditioned medium from cells expressing the PS1 L166P mutant, which has been reported to strongly promote Aβ42 generation (40), had much higher levels of Aβ42 than the medium from cells expressing WT PS1 (Fig. 3, *A*, *lower panel* and *B*). Most strikingly, SDS-PAGE analysis of the Nβ species in the same media (Fig. 3*A*, *upper panel*) showed that the major species was ~3 kDa (Nβ25) and the minor species was ~6 kDa (Nβ21), which is opposite the relative amounts in cells expressing WT PS1. MS analysis of the media of PS1 L166P expressing cells confirmed that the major secreted Nβ species are Nβ21 and Nβ25 with minor other species (Fig. 3*C*).

Therefore, PS1 L166P greatly enhanced not only the production of the long and pathological form of Aβ (Aβ42), but also the production of the longer form of Nβ (Nβ25) (Fig. 3, *A* and *C*). Likewise, the relative ratio of Nβ25 secreted from cells expressing PS1 L286V, PS1 Δ9, and other tested mutants except for C92S (39) increased compared with cells expressing WT PS1 (Fig. 3*B* and data not shown). We therefore suggest that S4-21 and S4-25 cleavage of Notch-1 are γ40- and γ42-like cleavage of βAPP, respectively (Fig. 3*D*).

Modifications of Intramembrane Proteolysis in an Endogenous WT PS Background Have Similar Effects on the Precision of S4 and γ-Cleavages—Recent findings suggest that the relative level of Aβ42 can be changed without mutations in PS or βAPP by using certain chemicals (29, 30). A subset of NSAIDs either increases (29) or decreases (30) the level of Aβ42 generation. Therefore, we finally examined whether, in an endogenous PS background, chemicals can cause parallel changes in the relative levels of Nβ25 and Aβ42. To lower Aβ42, we used sulindac sulfide (100 μM), indomethacin (100 μM) (29), and a newly developed compound, 3,5-bis(4-nitrophenoxy)-benzoic acid, which is called compound W (CW) (100 μM). CW was the most effective of ~100 tested compounds identified in a computer-based structural similarity search for NSAIDs that affect the production of Aβ42. The cells were treated with the compounds and then subjected to metabolic labeling with [^{35}S]methionine. The levels of Aβ/Nβ species were measured as described in Fig. 3. Remarkably, we found that the tested compounds all decreased the relative level of released Nβ25 as well as Aβ42 (Fig. 4, *A*, *left panels*, and *B*). Of these compounds, the most effective, CW, caused a drastic decrease in the level of secreted Nβ25 (Fig. 4*A*, *upper left panel*) and Aβ42 species (Fig. 4*A*, *middle* and *lower left panels*). Next, we examined the effects of S2474 (20 μM), fenofibrate (100 μM), farnesyl pyrophosphate ammonium salt (10 μM), and geranylgeranyl pyrophosphate ammonium salt (10 μM), which are compounds that raise the level of Aβ42 (30). As expected, these compounds all increased the relative level of Aβ42 (Fig. 4, *A*, *right panels*, and *B*). Notably, in all the cases analyzed, the relative level of Nβ25 generation was simultaneously increased (Fig. 4*B*). Naproxen did not modify the relative level of Aβ42 (29) or Nβ25 (Fig. 4, *B* and *C*). A plot of the ratio of Nβ25 to total Nβ *versus* the ratio of Aβ42 to total Aβ (Fig. 4*C*) showed a strong linear correlation (R^2 = 0.98) between the changes in the relative levels of Nβ25 and Aβ42. Moreover, the total level of Aβ and Nβ was not changed by any of the compounds, suggesting that they affect the selectivity but not the activity of cleavage (Fig. 4*A*). These results indicate that, even in cells expressing endogenous PS, the chemicals caused a concomitant change in the relative levels of Nβ25 and Aβ42.

DISCUSSION

In this study, we showed that Nβ species are secreted from cells as a result of sequential Notch-1 proteolysis during Notch signaling. Upon ligand binding, these peptides are likely generated by PS-dependent intramembrane endoproteolysis at S4 after shedding of the extracellular domain (cleavage at S2). Similarly, AD-associated Aβ species are produced by the PS-dependent proteolysis of βAPP (41, 42). Based on these findings, we suspect that cleavage at the middle of the transmembrane domain and extracellular secretion of Aβ-like peptides (*e.g.* Nβ) are a common occurrence in PS-dependent intramembrane proteolysis, even though secretion of such peptides is not included in the current model of RIP signaling.

The human *Notch-1* homologue, *TAN-1*, was isolated as a protein that binds to a mutant β T cell receptor gene formed by chromosomal translocation in human T lymphoblastic leukemia, suggesting that *Notch-1* is involved in tumorigenesis as well as development (8). Moreover, more than 50% of human T lymphoblastic leukemia, including tumors from all major molecular oncogenic subtypes, have activating mutations in Notch-1 (9). Thus, further studies are necessary to determine whether secreted Nβ participates in tumorigenesis or has any other physiological functions in development. In addition, it will be interesting to investigate whether the amount of secreted Nβ reflects the level of Notch signaling so that Nβ might be used as a tumor marker.

In this study, Nβ consisted mainly of a major Nβ21 species and a minor Nβ25 species, indicating that S4 cleavage occurs primarily at two sites, S4-21 and S4-25, in the middle of the Notch-1 transmembrane domain. This confirms that dual-intramembrane proteolysis occurs at S3 and S4 upon degradation of the transmembrane domain of Notch-1 (19). Moreover, this diversity in the sites of cleavage in the middle of the transmembrane domain is reminiscent of the mechanism producing Aβ42, a peptide that plays a causative role in AD (22). Therefore, we

very minor species of Nβ may be released in addition to Nβ21 and Nβ25. *D*, the relative ratio of Nβ25 to that of Nβ21 in the conditioned media of cells stably expressing N1CS. Cells were labeled with [^{35}S]methionine, and radiolabeled Nβ peptides in the conditioned medium were separated by Tris-Tricine SDS-PAGE. The radioactivity incorporated into each band was measured by fluorography using a STORM 820 (Amersham Biosciences). The radioactivity was normalized by that of the Nβ21 band. The *bars* represent the means of three independent measurements, and the *error bars* indicate the S.D. *E*, schematic representation of two different Nβ species and the S2, S3, S4 in Notch-1. The *gray box* indicates the putative transmembrane domain of murine Notch-1.

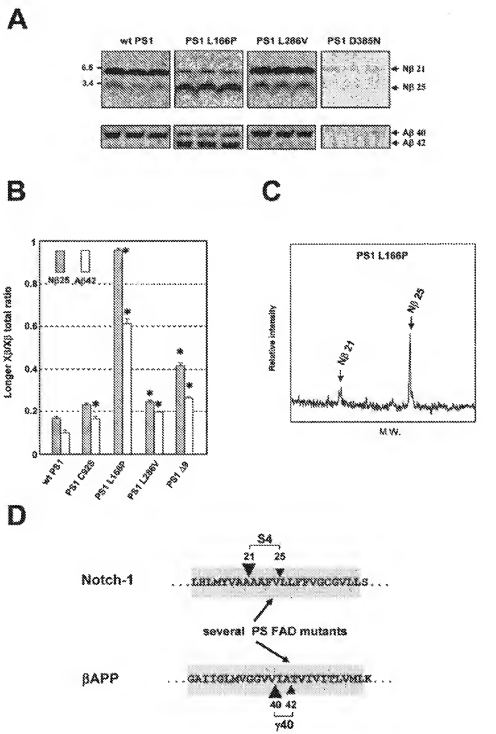

FIGURE 3. **The effects of PS1 mutants on the secretion of the longer Nβ.** *A*, Nβ and Aβ secreted from cells expressing PS1 mutants. *Upper panels*, Nβ species were analyzed as described in Figs. 1 and 2. *Lower panels*, Aβ was immunoprecipitated with antibody 4G8 and separated by Tris-Bicine SDS-PAGE. Note that Nβ and Aβ are hardly detectable in cells stably expressing PS1 D385N, which is dominant negative for PS-dependent protease activity. *B*, the relative ratio of longer Xβ (Nβ25 and Aβ42) secreted by cells expressing familial AD-associated PS mutants. Three independent experiments were carried out with each of two different stable cell lines. *Asterisks* indicate that the ratio of longer Xβ was statistically higher than that in WT PS1-expressing cells ($p < 0.001$ by Student's *t*-test). Similar results were obtained in CHO cells expressing familial AD-associated PS1 mutants (36). *C*, MS spectrum of Nβ species secreted from cells expressing the PS1 L166P mutant. The Nβ25 peak was much higher than the Nβ21 peak comparing the case of WT PS1 (see Fig. 2*A*). Note that the Nβ peptides secreted by the cells are mainly Nβ25 and Nβ21. *D*, schematic representation of the sites cleaved in Notch-1 and βAPP by PS-dependent intramembrane proteolysis during the generation of Xβ species. *Gray boxes* indicate the putative transmembrane domain in Notch-1 and βAPP. The *arrowheads* show the S4 and γ-cleavage sites. *Arrows* show the sites where the efficiency of cleavage is affected by tested familial AD-associated PS mutants.

tested whether the relationship between Nβ21 and Nβ25 was similar to that between Aβ40 and Aβ42. Most familial AD-associated PS mutants increase the generation of Aβ42 (22). Thus, we examined whether the PS mutants result in an extended C terminus in Nβ. We found that, except for the PS1 C92S mutant, the tested PS mutants increased the relative amount of Nβ25. One possible reason for this anomaly is that the mechanism by which the PS1 C92S mutant enhances Aβ42 generation affects Notch-1 processing differently. Another possibility is that the mutant is simply not strong enough to significantly increase the production of Nβ25. Moreover, the PS1 L166P mutants that greatly increased the level of Aβ42 also greatly increased the level of Nβ25. Therefore, in many cases, PS1 mutants that affect the precision of intramembrane proteolysis of βAPP have similar effects on Notch-1 cleavage.

Aβ42 accounts for ~10% of the total Aβ in cells expressing endogenous PS (22). Recently, several NSAIDs were found to either increase or decrease the relative production of Aβ42 (29, 30). This is extremely important because it shows that, as in sporadic AD, the precision of γ-cleavage can change without mutation in either in the substrate (βAPP) or the key enzyme component (PS). This prompted us to investigate whether such compounds also affect the C terminus of Nβ. Because NSAIDs affect the precision of γ-cleavage independently of its

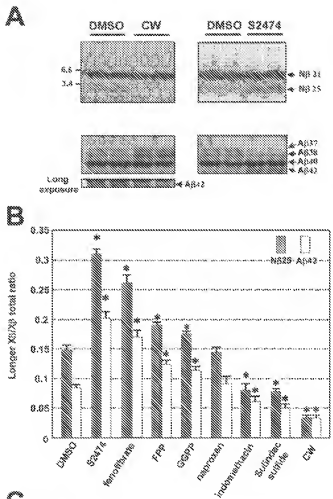

FIGURE 4. **Effects of compounds that modify the generation of Aβ42 on the secretion of longer Nβ25.** *A*, effect of various compounds on the secretion of Nβ and Aβ species from cells expressing βAPP sw and N1CS. Note that the decrease in the level of Aβ42 occurred concomitantly with an increase in the level of Aβ37/38 generation. Likewise, an increase in the level of Aβ42 was accompanied by a decrease in the level of Aβ37/38 generation. *DMSO*, Me$_2$SO. *B*, the relative ratio of longer Xβ (Nβ25 and Aβ42) secreted from endogenous PS-expressing cells treated with various compounds. *Asterisks* indicate that the relative ratio was statistically different from the Me$_2$SO control ($p < 0.001$ by Student's *t* test). *C*, a scatter plot of the relative ratio of Nβ25 versus the relative ratio of Aβ42. The same experiments as in *panel B* were independently repeated.

anti-inflammatory function (43), we performed a structure similarity search of Aβ42-lowering NSAIDs, and we identified CW as being the most effective of ~100 compounds. Strikingly, all of the tested compounds that affected the level of Aβ42 caused parallel changes in the generation of Nβ25. These results suggest that Nβ25 is the molecular species that corresponds to Aβ42 and that the PS-dependent protease generates longer Nβ and longer Aβ by a common mechanism.

Although increased production of Aβ42 is thought to cause the pathogenesis of most forms of familial AD, abnormalities in Aβ metabolism rather than increased Aβ42 generation are thought to be central to the pathogenesis of sporadic AD (44). Notably, the level of Aβ peptides, especially Aβ42, in peripheral blood and cerebrospinal fluid do not correlate with their levels in the central nervous system because they tend to aggregate. Therefore, whether Aβ42 generation changes in sporadic AD remains unknown. In this study, we showed that longer Aβ-like peptide species (Nβ25) and Aβ (Aβ42) are secreted concomitantly, which suggests that elongated Aβ-like peptides that do not aggregate could be used as surrogate markers for Aβ42 production.

In this study, we found that S4 and γ-cleavages, which occur in the middle of the transmembrane domain, have nearly identical characteristics. Also, S3 and ε cleavages, which occur near the cytosol-membrane interface, are very similar (40, 45).[4] Thus, PS-dependent intramembrane proteolysis may be mediated by a common mechanism in Notch-1 and βAPP. However, the processes mediating cleavage at ε and S3 seem to be distinct from that mediating γ-cleavage (40). Therefore, PS-dependent intramembrane proteolysis in the middle of the transmembrane domain (S4 and γ) and at the membrane-cytosol interface (ε and S3) appears to be mediated by distinct mechanisms.

The effect of NSAIDs on the precision of PS-dependent intramembrane proteolysis was thought to be βAPP-specific because the compounds affected precision of γ-cleavage but not the level of S3 cleavage (which generates NICD) (29). Our results, however, suggest that the effects of the compounds are not substrate-specific but may affect S4- and γ-like cleavages that occur near the middle of the transmembrane domains.

Comparison of the processes and precision of S4 and γ cleavage should help clarify how Aβ and Aβ42, essential molecules in AD pathology, are generated. The βAPP γ-cleavage shares the following characteristics with Notch-1 S4 cleavage. (i) Both of the cleavages occur near the middle of the transmembrane domain (19). (ii) Both have alternative cleavage sites (see Fig. 3*D*). (iii) Several familial AD-associated PS mutations enhance the cleavage efficiencies at minor C-terminal sites of both peptides (S4-25 and γ42). (iv) Several compounds simultaneously change both S4-25 and γ42 cleavage efficiencies. Although the amino acid sequences of the transmembrane domains of Notch-1 and βAPP are quite different, they are thought to both contain α-helical structures (see Fig. 3*D*). Therefore, the common aspects of S4 and γ-cleavage do not appear to be due to similarities in the primary structure of the substrate (transmembrane domains of Notch-1 and βAPP) but rather similarities in the secondary structures or higher structures of the enzyme-substrate complex.

Although there are significant similarities in the two cleavages, there are also some minor differences as follows. (i) The major γ-site is almost in the center of the transmembrane domain of βAPP, whereas the corresponding site in Notch-1, S4-21, is located slightly toward the extracellular side in the transmembrane domain (Fig. 3*D*). (ii) The distance between the major γ40 and the minor γ42 site is two amino acids, whereas the distance between S4-21 and S4-25 is four amino acids (Figs. 2*E* and 3*D*). (iii) The cleavage efficiency at γ42 is ~10%, whereas that of S4-25 is ~20% (Fig. 2*D*). These distinct properties are likely to be at least partly because of differences in the primary structures of the substrates. Comparison of the characteristics of S4 and γ-cleavage should help

[4] A. Fukumori, S. Tagami, and M. Okochi, submitted for publication.

clarify the process of Aβ42 generation; specifically, it appears that the peptide secondary or the higher structure of the enzyme-substrate complex is involved in the efficiency of Aβ42 generation, whereas the primary structure of the substrate transmembrane domain appears to determine the site of the minor cleavage.

When cleaved by PS-dependent intramembrane proteolysis, the βAPP transmembrane domain is thought to maintain an α-helical conformation (46). In this case, the pathological γ42 cleavage would occur in the opposite orientation as the major γ40 cleavage. In contrast, although the corresponding S4–25 site is located more toward the cytosolic side of the transmembrane domain, it is in nearly the same orientation at the S4–21 site. Thus, assuming α-helical structures of the substrate, cleavage at S4–25 and γ42 would be expected to occur by different mechanisms. Because there are so many similarities between the two, we propose that until the cleavage, perhaps in the enzyme-substrate complex, the secondary structures of the substrate transmembrane domains change so that they are no longer α-helices.

In this study we showed that Nβ species (mainly Nβ21 and Nβ25) are released extracellularly during the process of Notch signaling. The Nβ species had identical N termini but different C termini because of diversity in the site of S4 cleavage. Furthermore, the characteristics of the C-terminal elongation of Nβ and Aβ were almost identical. Because many membrane-bound receptors undergo PS-dependent intramembrane proteolysis, we propose that secretion of Aβ-like peptides such as Nβ may be a common phenomenon. This should open the door to a search for the best Aβ-like peptide to serve as a surrogate marker for AD-associated production of Aβ42.

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